TREATMENT OF CORONARY DISORDERS USING TNFα INHIBITORS

RELATED APPLICATIONS

This application claims priority to prior filed U.S. Provisional Application Serial No. 60/397,275, filed July 19, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/411,081, filed September 16, 2002, and prior-filed U.S. Provisional Application Serial No. 60/417490, filed October 10, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/455777, filed March 18, 2003. In addition, this application is related to U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015. This application is also related to U.S. Patent Application Serial No. 10/302356, filed November 22, 2002; U.S. Patent Application Serial No. 09/801,185, filed March 7, 2001; U.S. Patent Application Serial No. 10/163657, filed June 2, 2002; and U.S. Patent Application Serial No. 10/133715, filed April 26, 2002.

This application is related to U.S. utility applications (Attorney Docket No. BPI-187) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-188) entitled "Treatment of Spondyloarthropathies Using TNFα Inhibitors," (Attorney Docket No. BPI-189) entitled "Treatment of Pulmonary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-190) entitled "Treatment of Coronary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-191) entitled "Treatment of Metabolic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-192) entitled "Treatment of Anemia Using TNFα Inhibitors," (Attorney Docket No. BPI-193) entitled "Treatment of Pain Using TNFα Inhibitors," (Attorney Docket No. BPI-194) entitled "Treatment of Hepatic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-195) entitled "Treatment of Skin and Nail Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-196) entitled "Treatment of Vasculitides Using TNFα Inhibitors," (Attorney Docket No. BPI-197) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," and PCT application (Attorney Docket No. BPI-187PC) entitled "Treatment of TNFα-Related Disorders," all of which are filed on

even date herewith. The entire contents of each of these patents and patent applications are hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Coronary artery disease is a major cause of morbidity and mortality in the Western world. The disease is typically manifested in intravascular stenosis (narrowing) or occlusion (blockage) due to atherosclerotic plaque. Percutaneous transluminal coronary balloon angioplasty (PTCA), for example, is widely used as the primary treatment for arteriosclerosis involving stenosis. PCTA is any percutaneous transluminal method of decreasing stenosis within a blood vessel. PTCA has an immediate success rate of more than 95%, but long term success remains limited by restenosis in 20-50% of patients within six months after intervention (Bult, H. (2000) *Trends in Pharmacological Sciences* 21:274-279). Stent implantation may improve the clinical outcome of PTCA, however, restenosis still remains a major clinical challenge.

Restenosis, the process of arterial re-narrowing, is a combination of neointimal formation and arterial remodeling in response to vascular injury, such as that resulting from PTCA or other initially successful intervention. Vascular remodeling has a significant impact on chronic lumen area and may be responsible for 50% to 90% of late luminal area loss (Kumar, *et al.* (1997) *Circulation* 96(12):4333-4342). Remodeling is an adaptive process that occurs in response to chronic changes in hemodynamic conditions and may involve changes in many processes, such as cell growth, cell death, cell migration, and changes in extracellular matrix composition, that lead to a compensatory adjustment in vessel diameter and lumen area. The blood vessel is thought to remodel itself in response to long-term changes in flow, such that the lumen area is modified to maintain a predetermined level of shear stress (Kumar, *et al.* (1997) *Circulation* 96(12):4333-4342 and Orrego, *et al.* (1999) *Cardiologia* 44(7)621). It is estimated that in about one-third of PTCA treatments, arterial blockage returns in the form of restenosis within six months. It is thought that restenosis is the immune system's response to the "injury" of the angioplasty.

Cytokines, such as TNFα, are produced by a variety of cells, and have been identified as mediators of inflammatory processes. TNFα (also known as TNF) is produced by numerous cell types, including monocytes and macrophages, that was

originally identified based on its capacity to induce the necrosis of certain mouse tumors (see *e.g.*, Old, L. (1985) *Science* 230:630-632). Cytokines regulate the intensity and duration of the inflammatory response which occurs as the result of an injury, disease or infection.

SUMMARY OF THE INVENTION

The present invention provides methods for treating coronary disease where TNFα activity is detrimental in a safe and effective manner. Excessive or unregulated TNFα production has been implicated in mediating a number of diseases, including cardiovascular diseases, such as restenosis. Patients suffering from coronary disease, have elevated levels of TNFα circulating in their blood. In a study of patients who had undergone repeated atherectomy followed by PTCA, there was an increased expression of TNF and fibronectin in restenotic lesions compared to primary lesions (Clausell *et al.* (1995) *Br Heart J* 73:534).

The invention provides a method of treating a subject suffering from a coronary disorder comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less, such that the coronary disorder is treated.

The invention also provides a method of treating a subject suffering from a coronary disorder comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4

by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

The invention also includes a method of treating a subject suffering from a coronary disorder comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In one embodiment the antibody, or antigen-binding fragment thereof, is D2E7, also referred to as HUMIRA® (adalimumab). In another embodiment, the coronary disorder is restenosis. In yet another embodiment, the coronary disorder is selected from the group consisting of acute congestive heart failure, an acute coronary syndrome (including angina and myocardial infarction), artherosclerosis, chronic artherosclerosis, cardiomyopathy, congestive heart failure (chronic and acute), and rheumatic heart disease.

In one embodiment, the invention describes a method of treating a subject suffering from restenosis comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less, such that said restenosis is treated.

In yet another embodiment, the invention includes a method of treating a subject suffering from restenosis comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4

by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

In still another embodiment, the invention describes a method of treating a subject suffering from restenosis comprising administering a therapeutically effective amount a TNFα antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In one embodiment the TNFα antibody, or antigen binding fragment thereof, is D2E7. In another embodiment, the TNFα antibody is administered with at least one additional therapeutic agent.

The invention also includes a method for inhibiting human TNFα activity in a human subject suffering from a coronary disorder comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. In one embodiment the coronary disorder is restenosis. In still another embodiment the coronary disorder is selected from the group consisting of acute congestive heart failure, an acute coronary syndrome (including angina and myocardial infarction), artherosclerosis, chronic artherosclerosis, cardiomyopathy, congestive heart failure (chronic and acute), and rheumatic heart disease. In still another embodiment, the TNFα antibody, or antigen-binding fragment thereof, is D2E7.

The invention includes a method for inhibiting human TNFα activity in a human subject suffering from restenosis, comprising administering a therapeutically effective amount of a TNFα antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. In one embodiment, the antibody, or antigen binding fragment thereof, is D2E7.

In one embodiment, the invention describes a method of treating a subject suffering from a coronary disorder comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that the coronary disorder is treated. In one embodiment, the coronary disorder is restenosis. In another embodiment, the coronary disorder is selected from the group consisting of acute congestive heart failure, an acute coronary syndrome (including angina and myocardial infarction), artherosclerosis, chronic artherosclerosis, cardiomyopathy, congestive heart failure (chronic and acute), and rheumatic heart disease.

In still another embodiment, the invention includes a method of treating a subject suffering from restenosis comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that said restenosis is treated.

The invention also describes a method of treating a subject suffering from or at risk of developing restenosis comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, and at least one additional therapeutic agent to the subject, such that the coronary disorder is treated. In one embodiment, the additional therapeutic agent is selected from the group consisting of sirolimus, paclitaxel, everolimus, tacrolimus, ABT-578, and acetaminophen.

In still another embodiment, the invention describes a kit comprising a pharmaceutical composition comprising a TNFα antibody, or an antigen binding portion thereof, and a pharmaceutically acceptable carrier; and instructions for administering to a subject the TNFα antibody pharmaceutical composition, for treating a subject who is suffering from a coronary disorder. In one embodiment, the TNFα antibody, or an antigen binding portion thereof, in the kit is D2E7.

DETAILED DESCRIPTION OF THE INVENTION

This invention pertains to methods of treating a coronary disorder in which TNFα activity, e.g., human TNFα activity, is detrimental. The methods include administering to the subject an effective amount of a TNFα antibody, such that the coronary disorder is treated or prevented. In one embodiment, the TNFα antibody of the invention is administered to treat or prevent restenosis. The invention also pertains to

methods wherein the TNFα inhibitor is administered in combination with another therapeutic agent to treat a coronary disorder. Various aspects of the invention relate to treatment with antibodies and antibody fragments, and pharmaceutical compositions comprising a TNFα inhibitor, and a pharmaceutically acceptable carrier for the treatment of a coronary disorder.

Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNFα" (abbreviated herein as hTNFα, or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNFα is described further in, for example, Pennica, D., *et al.* (1984) *Nature* 312:724-729; Davis, J.M., *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y., *et al.* (1989) *Nature* 338:225-228. The term human TNFα is intended to include recombinant human TNFα (rhTNFα), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN). TNFα is also referred to as TNF.

The term "TNFα inhibitor" includes agents which inhibit TNFα. Examples of TNFα inhibitors include etanercept (Enbrel®, Amgen), infliximab (Remicade®, Johnson and Johnson), human anti-TNF monoclonal antibody (D2E7/HUMIRA®, Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech) and other compounds which inhibit TNFα activity, such that when administered to a subject suffering from or at risk of suffering from a disorder in which TNFα activity is detrimental, the disorder is treated. In one embodiment, a TNFα inhibitor is a compound, excluding etanercept and infliximab, which inhibits TNFα activity. In another embodiment, the TNFα inhibitors of the invention are used to treat a TNFα-related disorder, as described in more detail in section II. In one embodiment, the TNFα inhibitor, excluding etanercept and infliximab, is used to treat a TNFα-related disorder. In another embodiment, the TNFα inhibitor, excluding etanercept and infliximab, is used to treat a coronary disorder. The term also

includes each of the anti-TNFα human antibodies and antibody portions described herein as well as those described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356.

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The antibodies of the invention are described in further detail in U.S. Patent Nos. 6,090,382; 6,258,562; and 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using

recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123). The antibody portions of the invention are described in further detail in U.S. Patent Nos. 6,090,382, 6,258,562, 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.
Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies. Other than "bispecific" or "bifunctional" immunoglobulins or antibodies, an immunoglobulin or antibody is understood to have each of its binding sites identical. A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, Clin. Exp. Immunol. 79:315-321 (1990); Kostelny et al., J. Immunol. 148, 1547-1553 (1992).

A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-

branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα). An isolated antibody that specifically binds hTNFα may, however, have cross-reactivity to other antigens, such as hTNFα

molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody", as used herein (or an "antibody that neutralized hTNFα activity"), is intended to refer to an antibody whose binding to hTNFα results in inhibition of the biological activity of hTNFα. This inhibition of the biological activity of hTNFα can be assessed by measuring one or more indicators of hTNFα biological activity, such as hTNFα-induced cytotoxicity (either *in vitro* or *in vivo*), hTNFα-induced cellular activation and hTNFα binding to hTNFα receptors. These indicators of hTNFα biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see U.S. Patent No. 6,090,382). Preferably, the ability of an antibody to neutralize hTNFα activity is assessed by inhibition of hTNFα-induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNFα activity, the ability of an antibody to inhibit hTNFα-induced expression of ELAM-1 on HUVEC, as a measure of hTNFα-induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnsson, B., *et al.* (1995) *J. Mol. Recognit.* 8:125-131; and Johnnson, B., *et al.* (1991) *Anal. Biochem.* 198:268-277.

The term "K_{off}", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_d", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "IC_{50}" as used herein, is intended to refer to the concentration of the inhibitor required to inhibit the biological endpoint of interest, e.g., neutralize cytotoxicity activity.

The term “nucleic acid molecule”, as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term “isolated nucleic acid molecule”, as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNFα, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-hTNFα antibody contains no other sequences encoding other VH regions that bind antigens other than hTNFα.

The term “vector”, as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, “expression vectors”). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term “recombinant host cell” (or simply “host cell”), as used herein, is intended to refer to a cell into which a recombinant expression vector has been

introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The term "dosing", as used herein, refers to the administration of a substance (*e.g.*, an anti-TNFα antibody) to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder).

The terms "biweekly dosing regimen", "biweekly dosing", and "biweekly administration", as used herein, refer to the time course of administering a substance (*e.g.*, an anti-TNFα antibody) to a subject to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder). The biweekly dosing regimen is not intended to include a weekly dosing regimen. Preferably, the substance is administered every 9-19 days, more preferably, every 11-17 days, even more preferably, every 13-15 days, and most preferably, every 14 days.

The term "combination" as in the phrase "a first agent in combination with a second agent" includes co-administration of a first agent and a second agent, which for example may be dissolved or intermixed in the same pharmaceutically acceptable carrier, or administration of a first agent, followed by the second agent, or administration of the second agent, followed by the first agent. The present invention, therefore, includes methods of combination therapeutic treatment and combination pharmaceutical compositions.

The term "concomitant" as in the phrase "concomitant therapeutic treatment" includes administering an agent in the presence of a second agent. A concomitant therapeutic treatment method includes methods in which the first, second, third, or additional agents are co-administered. A concomitant therapeutic treatment method also includes methods in which the first or additional agents are administered in the presence of a second or additional agents, wherein the second or additional agents, for example, may have been previously administered. A concomitant therapeutic treatment method may be executed step-wise by different actors. For example, one actor may administer to a subject a first agent and a second actor may to administer to the subject a second agent, and the administering steps may be executed at the same time, or nearly the same

time, or at distant times, so long as the first agent (and additional agents) are after administration in the presence of the second agent (and additional agents). The actor and the subject may be the same entity (*e.g.*, human).

The term "combination therapy", as used herein, refers to the administration of two or more agents, *e.g.*, an anti-TNFα antibody and another drug, such as a DMARD or NSAID. The other drug(s) may be administered concomitant with, prior to, or following the administration of an anti-TNFα antibody.

The term "kit" as used herein refers to a packaged product comprising components with which to administer the TNFα antibody of the invention for treatment of a TNFα-related disorder. The kit preferably comprises a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which are preferably contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering the TNFα antibody of the invention.

The term "cardiovascular disorder" or "coronary disorder" as used interchangeably herein, refers to any disease, disorder, or state involving the cardiovascular system, e.g., the heart, the blood vessels, and/or the blood. A coronary disorder is generally characterized by a narrowing of the blood vessels that supply blood and oxygen to the heart (coronary arteries). Coronary disease usually results from the build up of fatty material and plaque. As the coronary arteries narrow, the flow of blood to the heart can slow or stop. Coronary disorders of the invention can apply to any abnormality of an artery, whether structural, histological, biochemical or any other abnormality. An example of coronary heart disease is restenosis. In one embodiment, a coronary disorder refers to any disease, disorder, or state involving the cardiovascular system excluding ischemia of the heart and heart insufficiency.

The term "restenosis" as used herein refers to the recurrence of stenosis, which is the narrowing or constriction of an artery. Restenosis often occurs as a preocclusive lesion that develops following a reconstructive procedure in a diseased blood vessel. The term is not only applied to the recurrence of a pre-existing stenosis, but also to previously normal vessels that become partially occluded following vascular bypass. In another embodiment, the invention provides a method of treating restenosis comprising

administering the antibody, or antigen binding portion thereof, of the invention to a subject who has or is at risk of developing restenosis.

The term "stent" as used herein refers to a structure that is inserted into the lumen of an anatomical vessel, *e.g.* an artery, especially to keep a formerly blocked passageway open. Stent is used to maintain the flow of fluids (e.g., blood) from one portion of a vessel to another, and an endovascular scaffolding or stent which holds open a body passageway and/or supports the graft or wrap. A stent is often used following balloon angioplasty, although they can also be used as direct therapy for treating stenosis.

In one embodiment of the invention, the stent is drug-eluting. The term "drug-eluting" refers to a stent which is coated with a slow-to-moderate release drug formulation. The terms "drug-eluting" or "drug-releasing" or "drug-coated" are used interchangeably herein. A stent can be coated with any drug which treats coronary heart disease, including, for example, the antibody, or antigen-binding fragment thereof, of the invention. In another embodiment, the stent delivers D2E7. In a further embodiment, the stent delivers D2E7 in combination with another drug used to treat coronary disorders, including dexamethasone, alkeran, cytoxan, leukeran, cis-platinum, BiCNU, adriamycin, doxorubicin, cerubidine, idamycin, mithracin, mutamycin, fluorouracil, methotrexate, thoguanine, toxotere, etoposide, vincristine, irinotecan, hycamptin, matulane, vumon, hexalin, hydroxyurea, gemzar, oncovin, etophophos, tacrolimus (FK506), and the following analogs of sirolimus: SDZ-RAD, CCI-779, 7-epi-rapamycin, 7-thiomethyl-rapamycin, 7-epi-trimethoxyphenyl-- rapamycin, 7-epi-thiomethyl-rapamycin, 7-demethoxy-rapamycin, 32-demethoxy, 2-desmethyl and proline.

Various aspects of the invention are described in further detail herein.

I. <u>TNFα Inhibitors of the Invention</u>

This invention provides methods of treating cardiovascular disorders in which the administration of a TNFα inhibitor is beneficial. In one embodiment, these methods includes administration of isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNFα antibodies. The most preferred recombinant,

neutralizing antibody of the invention is referred to herein as D2E7 (the amino acid sequence of the D2E7 VL region is shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is shown in SEQ ID NO: 2). D2E7 is also referred to as HUMIRA® and adalimumab. The properties of D2E7 have been described in Salfeld *et al.*, U.S. patent No. 6,090,382, which is incorporated by reference herein.

In one embodiment, the treatment of the invention includes the administration of D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides treatment with an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of $1 \times 10^{-3}\ s^{-1}$ or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of $5 \times 10^{-4}\ s^{-1}$ or less, or even more preferably. with a K_{off} of $1 \times 10^{-4}\ s^{-1}$ or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10^{-9} M or less and still more preferably with an IC_{50} of 1×10^{-10} M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof.

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to methods of treating disorders in which the TNFα activity is detriment by administering human antibodies that have slow dissociation kinetics for association with hTNFα and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. Position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the

D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off}. Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNFα. Positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNFα and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above) (*see* U.S. Patent No. 6,090,382).

Accordingly, in another embodiment, the invention provides methods of treating cardiovascular disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains the following characteristics:

a) dissociates from human TNFα with a K_{off} rate constant of $1 \times 10^{-3}\ s^{-1}$ or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2,

3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of $5 \times 10^{-4}\ s^{-1}$ or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of $1 \times 10^{-4}\ s^{-1}$ or less.

In yet another embodiment, the invention provides methods of treating cardiovascular disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably are from the $V_{\kappa}I$ human germline family, more preferably from the A20 human germline Vk gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B of U.S. Patent No. 6,090,382. The framework regions for VH preferably are from the V_H3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B U.S. Patent No. 6,090,382.

Accordingly, in another embodiment, the invention provides methods of treating cardiovascular disorders by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region

(HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides methods of treating cardiovascular disorders in which the administration of an anti-TNFα antibody is beneficial administration of an isolated human antibody, or an antigen-binding portions thereof. The antibody or antigen-binding portion thereof preferably contains D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In another embodiment, the TNFα inhibitor of the invention is etanercept (described in WO 91/03553 and WO 09/406476), infliximab (described in U.S. Patent No. 5,656,272), CDP571 (a humanized monoclonal anti-TNF-alpha IgG4 antibody), CDP 870 (a humanized monoclonal anti-TNF-alpha antibody fragment), D2E7/HUMIRA® (a human anti-TNF mAb), soluble TNF receptor Type I, or a pegylated soluble TNF receptor Type I (PEGs TNF-R1).

The TNFα antibody of the invention can be modified. In some embodiments, the TNFα antibody or antigen binding fragments thereof, is chemically modified to provide a desired effect. For example, pegylation of antibodies and antibody fragments of the invention may be carried out by any of the pegylation reactions known in the art, as

described, for example, in the following references: *Focus on Growth Factors* 3:4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of the antibodies and antibody fragments of the invention is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol.

Methods for preparing pegylated antibodies and antibody fragments of the invention will generally comprise the steps of (a) reacting the antibody or antibody fragment with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby the antibody or antibody fragment becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

Pegylated antibodies and antibody fragments may generally be used to treat coronary disorders by administration of the TNFα antibodies and antibody fragments described herein. Generally the pegylated antibodies and antibody fragments have increased half-life, as compared to the nonpegylated antibodies and antibody fragments. The pegylated antibodies and antibody fragments may be employed alone, together, or in combination with other pharmaceutical compositions.

In yet another embodiment of the invention, TNFα antibodies or fragments thereof can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see *e.g.*, Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on

FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (e.g., another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNFα antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V_{78} Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the $V_\kappa I$ family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31

germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab

fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* <u>242</u>:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* <u>85</u>:5879-5883; McCafferty *et al.*, *Nature* (1990) <u>348</u>:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one

approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host

cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above

procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNFα. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNFα by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

Recombinant human antibodies of the invention in addition to D2E7 or an antigen binding portion thereof, or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating

and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982. Methods of isolating human antibodies with high affinity and a low off rate constant for hTNFα are described in U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015, each of which is incorporated by reference herein.

II. Uses of TNFα Inhibitors of the Invention

The invention features a method for treating or preventing a coronary disorder in which TNFα activity is detrimental, comprising administering to a subject an effective amount of a TNFα inhibitor, such that said disorder is treated or prevented. In one embodiment, the coronary disorder is restenosis, acute congestive heart failure, an acute coronary syndrome (including angina and myocardial infarction), artherosclerosis, chronic artherosclerosis, cardiomyopathy, congestive heart failure (chronic and acute), and rheumatic heart disease. In one embodiment, the TNFα inhibitor is D2E7, also referred to as HUMIRA® (adalimumab).

In an embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from a cardiovascular disorder in which TNFα activity is detrimental. TNFα has been implicated in the pathophysiology of a wide variety of cardiovascular disorders, including restenosis (see *e.g.*, Clausell *et al.* (1994), *supra*; Medall *et al.* (1997) *Heart* 78(3):273). The invention provides methods for TNFα activity in a subject suffering from such a disorder, which method comprises

administering to the subject an antibody, antibody portion, or other TNFα inhibitor of the invention such that TNFα activity in the subject with or at risk of having restenosis, is inhibited. Preferably, the TNFα is human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (*e.g.*, by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages and time courses of administration).

As used herein, the term "a coronary disorder in which TNFα activity is detrimental" is intended to include coronary and cardiovascular diseases in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder, including cardiovascular disorders, *e.g.*, restenosis. Coronary disorders in which TNFα activity often results from a blockage in an artery. Such a blockage can be caused by a clot, which usually forms in a coronary artery that has been previously narrowed from changes usually related to atherosclerosis. For example, if the atherosclerotic plaque inside the arterial wall cracks, it can trigger the formation of a thrombus, or clot. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNFα in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. A coronary disorder can be also caused by an imbalance in arterial pressure, a malfunction of the heart, or an occlusion of a blood vessel, e.g., by a thrombus. Coronary disorders includes both coronary artery disease and peripheral vascular disease.

There are numerous examples of coronary disorders in which TNFα activity is detrimental, including restenosis. The use of the antibodies, antibody portions, and other TNFα inhibitors of the invention in the treatment of specific coronary disorders are discussed further below. In certain embodiments, the antibody, antibody portion, or other TNFα inhibitor of the invention is administered to the subject in combination with another therapeutic agent, as described below

The invention provides a method for inhibiting TNFα activity in a subject with a coronary disorder. The invention provides methods for inhibiting or decreasing TNFα activity in a subject with a coronary disorder, comprising administering to the subject an antibody, or antibody portion, or other TNFα inhibitor of the invention such that TNFα activity in the subject is inhibited or decreased. Preferably, the TNFα is human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (*e.g.*, by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages and time courses of administration).

Commonly used animal models for studying coronary disorders, including restenosis, include the rat or mouse carotid artery ligation model and the carotid artery injury model (Ferns *et al.* (1991) *Science* 253:1129; Clowes *et al.* (1983) Lab. Invest. 49:208; Lindner *et al.* (1993) *Circ Res.* 73:792). In the carotid artery ligation model, arterial blood flow is disrupted by ligation of the vessel near the distal bifurnation. As described in Clowes *et al.*, the carotid artery injury model is performed such that the common carotid artery is denuded of endothelium by the intraluminal passage of a balloon catheter introduced through the external carotid artery. At 2 weeks, the carotid artery is markedly narrowed due to smooth muscle cell constriction, but between 2 and

12 weeks the intimal doubles in thickness leading to a decrease in luminal size. Any of these models can be used to determine the potential therapeutic action of the TNFα antibodies of the invention in the prevention and treatment of restenosis in humans.

The antibody of the invention can be used to treat cardiovascular disorders in which TNFα activity is detrimental, wherein inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the coronary disease or to prevent the coronary disease. Subjects suffering from or at risk of developing coronary disorders can be identified through clinical symptoms. Clinical symptoms in coronary disease often include chest pain, shortness of breath, weakness, fainting spells, alterations in consciousness, extremity pain, paroxysmal nocturnal dyspnea, transient ischemic attacks and other such phenomena experienced by the patient. Clinical signs of coronary disease can also include EKG abnormalities, altered peripheral pulses, arterial bruits, abnormal heart sounds, rates and wheezes, jugular venous distention, neurological alterations and other such findings discerned by the clinician. Coronary disorders may also be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNFα in serum, plasma, synovial fluid, etc. of the subject).

Examples of a cardiovascular disorder include, but are not limited to, coronary artery disease, angina pectoris, myocardial infarction, cardiovascular tissue damage caused by cardiac arrest, cardiovascular tissue damage caused by cardiac bypass, cardiogenic shock, and hypertension, atherosclerosis, coronary artery spasm, coronary artery disease, valvular disease, arrhythmias, and cardiomyopathies. The use of the antibodies, antibody portions, and other TNFα inhibitors of the invention in the treatment of specific cardiovascular diseases are discussed further below. In certain embodiments, the antibody, antibody portion, or other TNFα inhibitor of the invention is administered to the subject in combination with another therapeutic agent, as described below in section III.

A. *Restenosis*

TNFα has been implicated in the pathophysiology of restenosis (see Zhou *et al.* (2002) Atherosclerosis. 161:153; Javed *et al.* (2002) *Exp and Mol Pathol* 73:104). For

example, in the murine wire carotid model, TNF -/- mice demonstrated a seven-fold reduction in intial hyperplasia compared to wild type mice (Zimmerman *et al.* (2002) *Am J Phsiol Regul Integr Comp* Physiol 283:R505). Restenosis can occur as the result of any type of vascular reconstruction, whether in the coronary vasculature or in the periphery (Colburn and Moore (1998) Myointimal Hyperplasia pp. 690-709 in *Vascular Surgery: A Comprehensive Review* Philadelphia: Saunders). For example, studies have reported symptomatic restenosis rates of 30-50% following coronary angioplasties (see Berk and Harris (1995) *Adv. Intern. Med.* 40:455-501). After carotid endarterectomies, as a further example, 20% of patients studied had a luminal narrowing greater than 50% (Clagett *et al.* (1986) J. Vasc. Surg. 3:10-23). Restenosis is evidenced in different degrees of symptomatology which accompany preocclusive lesions in different anatomical locations, due to a combination of factors including the nature of the vessels involved, the extent of residual disease, and local hemodynamics.

"Stenosis," as used herein refers to a narrowing of an artery as seen in occlusive disorder or in restenosis. Stenosis can be accompanied by those symptoms reflecting a decrease in blood flow past the narrowed arterial segment, in which case the disorder giving rise to the stenosis is termed a disease (i.e., occlusive disease or restenosis disease). Stenosis can exist asymptomatically in a vessel, to be detected only by a diagnostic intervention such as an angiography or a vascular lab study.

The antibody of the invention can be used to treat a subject suffering from or at risk of developing restenosis. A subject at risk of developing restenosis includes a subject who has undergone PTCA. The subject may have also had a stent inserted to prevent restenosis. The TNFα antibody of the invention can be used alone or in combination with a stent to prevent the re-occurrence of stenosis in a subject suffering from cardiovascular disease.

B. *Congestive Heart Failure*

TNFα has been implicated in the pathophysiology of congestive heart failure (see Zhou *et al.* (2002) *Atherosclerosis* 161:153). Serum levels of TNFα are elevated in patients with congestive heart failure in a manner which is directly proportional to the severity of the disease (Levine *et al.* (1990) *N Engl J* Med 323:236; Torre-Amione *et al.* (1996) *J Am Coll Cardiol* 27:1201). In addition, inhibitors of TNFα have also been

shown to improve congestive heart failure symptoms (Chung *et al.* (2003) Circulation 107:3133).

As used herein, the term "congestive heart failure" includes a condition characterized by a diminished capacity of the heart to supply the oxygen demands of the body. Symptoms and signs of congestive heart failure include diminished blood flow to the various tissues of the body, accumulation of excess blood in the various organs, *e.g.*, when the heart is unable to pump out the blood returned to it by the great veins, exertional dyspnea, fatigue, and/or peripheral edema, *e.g.*, peripheral edema resulting from left ventricular dysfunction. Congestive heart failure may be acute or chronic. The manifestation of congestive heart failure usually occurs secondary to a variety of cardiac or systemic disorders that share a temporal or permanent loss of cardiac function. Examples of such disorders include hypertension, coronary artery disease, valvular disease, and cardiomyopathies, e.g., hypertrophic, dilative, or restrictive cardiomyopathies.

A "subject who has or is suffering from congestive heart failure" is a subject who has a disorder involving a clinical syndrome of diverse etiologies linked by the common denominator of impaired heart pumping in which the heart cannot pump blood commensurate with the requirements of the metabolizing tissues, or can do so only from an elevated filling pressure. A "subject at risk of developing congestive heart failure" is a subject who has a propensity of developing congestive heart failure because of certain factors affecting the cardiovascular system of the subject. It is desirable to reduce the risk of or prevent the development of congestive heart failure in these subjects. The phrase "with congestive heart failure" includes patients who are at risk of suffering from this condition relative to the general population, even though they may not have suffered from it yet, by virtue of exhibiting risk factors. For example, a patient with untreated hypertension may not have suffered from congestive heart failure, but is at risk because of his or her hypertensive condition. In one embodiment of the invention, the antibody D2E7 is used to treat a subject at risk of developing congestive heart failure.

C. *Acute coronary syndromes*

TNFα has been implicated in the pathophysiology of acute coronary syndromes (see Libby (1995) *Circulation* 91:2844). Acute coronary syndromes include those

disorders wherein the subject experiences pain due to a blood flow restriction resulting in not enough oxygen reaching the heart. Studies have found that TNFα plays a role in acute coronary syndromes. For example, in a novel rat heterotropic cardiac transplantation-coronary ligation model capable of inducing myocardial infarction in the absence of downstream hemodynamic effects, administration of chimeric soluble TNF receptor (sTNFR) abolished transient LV remodeling and dysfunction (Nakamura, *et al.* (2003) *J. Cardiol.* 41:41). It was also found that direct injection of an sTNFR expression plasmid to the myocardium, resulted in a reduction in the infarction size in acute myocardial infarction (AMI) experimental rats (Sugano *et al.* (2002) *FASEB J* 16:1421).

In one embodiment, TNFα antibody of the invention is used to treat or prevent an acute coronary syndrome in a subject, wherein the acute coronary syndrome is a myocardial infarction or angina.

As used herein, the term "myocardial infarction" or "MI" refers to a heart attack. A myocardial infarction involves the necorsis or permanent damage of a region of the heart due to an inadequate supply of oxygen to that area. This necrosis is typically caused by an obstruction in a coronary artery from either atherosclerosis or an embolis. MIs which are treated by the TNFα antibody of the invention include both Q-wave and non-Q-wave myocardial infarction. Most heart attacks are caused by a clot that blocks one of the coronary arteries (the blood vessels that bring blood and oxygen to the heart muscle). For example, a clot in the coronary artery interrupts the flow of blood and oxygen to the heart muscle, leading to the death of heart cells in that area. The damaged heart muscle permanently loses its ability to contract, and the remaining heart muscle needs to compensate for it. An MI can also be caused by overwhelming stress in the individual.

The term "angina" refers to spasmodic, choking, or suffocative pain, and especially as denoting angina pectoris which is a paroxysmal thoracic pain due, most often, to anoxia of the myocardium. Angina includes both variant angina and exertional angina. A subject having angina has ischemic heart disease which is manifested by sudden, severe, pressing substemal pain that often radiates to the left shoulder and along the left arm. TNFα has been implicated in angina, as TNFα levels are upregulated in patients with both MI and stable angina (Balbay *et al.* (2001) *Angiology* 52109).

D. *Artherosclerosis*

"Atherosclerosis" as used herein refers to a condition in which fatty material is deposited along the walls of arteries. This fatty material thickens, hardens, and may eventually block the arteries. Atherosclerosis is also referred to arteriosclerosis, hardening of the arteries, and arterial plaque buildup. Polyclonal antibodies directed against TNFα have been shown to be effective at neutralizing TNFα activity resulting in inflammation and restenosis in the rabbit atherosclerotic model (Zhou *et al.*, *supra*). Accordingly, the TNFα antibody of the invention can be used to treat or prevent subjects afflicted with or at risk of having atherosclerosis.

E. *Cardiomyopathy*

The term "cardiomyopathy" as used herein is used to define diseases of the myocardium wherein the heart muscle or myocardium is weakened, usually resulting in inadequate heart pumping. Cardiomyopathy can be caused by viral infections, heart attacks, alcoholism, long-term, severe hypertension (high blood pressure), or by autoimmune causes..

In approximately 75-80% of heart failure patients coronary artery disease is the underlying cause of the cardiomyopathy and is designated "ischemic cardiomyopathy." Ischemic cardiomyopathy is caused by heart attacks, which leave scars in the heart muscle or myocardium. The affected myocardium is then unable to contribute to the heart pumping function. The larger the scars or the more numerous the heart attacks, the higher the chance there is of developing ischemic cardiomyopathy.

Cardiomyopathies that are not attributed to underlying coronary artery disease, and are designated "non-ischemic cardiomyopathies." Non-ischemic cardiomyopathies include, but are not limited to idiopathic cardiomyopathy, hypertrophic cardiomyopathy, alcoholic cardiomyopathy, dilated cardiomyopathy, peripartum cardiomyopathy, and restrictive cardiomyopathy.

It is understood that all of the above-mentioned disorders include both the adult and juvenile forms of the disease where appropriate. It is also understood that all of the

above-mentioned disorders include both chronic and acute forms of the disease wherein appropriate. In addition, the TNFα antibody of the invention can be used to treat each of the above-mentioned TNFα-related disorders alone or in combination with one another.

III. Pharmaceutical Compositions and Pharmaceutical Administration

A. Compositions and Administration

The antibodies, antibody-portions, and other TNFα inhibitors of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody, antibody portion, or other TNFα inhibitor of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody, antibody portion, or other TNFα inhibitor.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies or other TNFα inhibitors. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody or other TNFα

inhibitor is administered by intravenous infusion or injection. In another preferred embodiment, the antibody or other TNFα inhibitor is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody, antibody portion, or other TNFα inhibitor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents. For example, an anti- hTNFα antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more DMARD or one or more NSAID or one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751) or any combination thereof. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination

therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

In one embodiment, the invention includes pharmaceutical compositions comprising an effective amount of a TNFα inhibitor and a pharmaceutically acceptable carrier, wherein the effective amount of the TNFα inhibitor may be effective to treat an coronary disorder, including restenosis.

The antibodies, antibody-portions, and other TNFα inhibitors of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

The TNFα antibodies of the invention can also be administered in the form of protein crystal formulations which include a combination of protein crystals encapsulated within a polymeric carrier to form coated particles. The coated particles of the protein crystal formulation may have a spherical morphology and be microspheres of up to 500 micro meters in diameter or they may have some other morphology and be microparticulates. The enhanced concentration of protein crystals allows the antibody of the invention to be delivered subcutaneously. In one embodiment, the TNFα antibodies of the invention are delivered via a protein delivery system, wherein one or more of a protein crystal formulation or composition, is administered to a subject with a TNFα-related disorder. Compositions and methods of preparing stabilized formulations of whole antibody crystals or antibody fragment crystals are also described in WO 02/072636, which is incorporated by reference herein. In one embodiment, a formulation comprising

the crystallized antibody fragments described in Examples 5 and 6 and are used to treat a TNFα-related disorder.

In certain embodiments, an antibody, antibody portion, or other TNFα inhibitor of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody, antibody portion, or other TNFα inhibitor may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody, antibody portion, other TNFα inhibitor to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, antibody portion, or other TNFα inhibitor are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used

herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 10-100 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The invention also pertains to packaged pharmaceutical compositions or kits which comprise a TNFα inhibitor of the invention and instructions for using the inhibitor to treat a particular disorder in which TNFα activity is detrimental, as described above. The package or kit alternatively can contain the TNFα inhibitor and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged pharmaceuticals or kits further can include a second agent (as described herein) packaged with or copromoted with instructions for using the second agent with a first agent (as described herein).

B. Additional therapeutic agents

The invention pertains to pharmaceutical compositions and methods of use thereof for the treatment of inflammatory disorders, including coronary disorders. The pharmaceutical compositions comprise a first agent that prevents or inhibits a coronary disorder. The pharmaceutical composition also may comprise a second agent that is an

active pharmaceutical ingredient; that is, the second agent is therapeutic and its function is beyond that of an inactive ingredient, such as a pharmaceutical carrier, preservative, diluent, or buffer. The second agent may be useful in treating or preventing coronary disease. The second agent may diminish or treat at least one symptom(s) associated with the targeted disease. The first and second agents may exert their biological effects by similar or unrelated mechanisms of action; or either one or both of the first and second agents may exert their biological effects by a multiplicity of mechanisms of action. A pharmaceutical composition may also comprise a third compound, or even more yet, wherein the third (and fourth, etc.) compound has the same characteristics of a second agent.

It should be understood that the pharmaceutical compositions described herein may have the first and second, third, or additional agents in the same pharmaceutically acceptable carrier or in a different pharmaceutically acceptable carrier for each described embodiment. It further should be understood that the first, second, third and additional agent may be administered simultaneously or sequentially within described embodiments. Alternatively, a first and second agent may be administered simultaneously, and a third or additional agent may be administered before or after the first two agents.

The combination of agents used within the methods and pharmaceutical compositions described herein may have a therapeutic additive or synergistic effect on the condition(s) or disease(s) targeted for treatment. The combination of agents used within the methods or pharmaceutical compositions described herein also may reduce a detrimental effect associated with at least one of the agents when administered alone or without the other agent(s) of the particular pharmaceutical composition. For example, the toxicity of side effects of one agent may be attenuated by another agent of the composition, thus allowing a higher dosage, improving patient compliance, and improving therapeutic outcome. The additive or synergistic effects, benefits, and advantages of the compositions apply to classes of therapeutic agents, either structural or functional classes, or to individual compounds themselves.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic

agents that are useful for treating inflammatory disorders in which TNFα activity is detrimental, including inflammatory disorders such as coronary disorders. For example, an anti-hTNFα antibody, antibody portion, or other TNFα inhibitor of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies or other TNFα inhibitors of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies. Specific therapeutic agent(s) are generally selected based on the particular disorder being treated, as discussed below.

Nonlimiting examples of therapeutic agents with which an antibody, antibody portion, or other TNFα inhibitor of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2/infliximab (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see *e.g.*, *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNF (soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol.

268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitos of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten

Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca *et al.* (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); azaribine; methotrexate; antivirals; and immune modulating agents. Any of the above-mentioned agents can be administered in combination with the TNFα antibody of the invention to treat a coronary disease, including restenosis.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of rheumatoid arthritis: methotrexate; prednisone; celecoxib; folic acid; hydroxychloroquine sulfate; rofecoxib; etanercept; infliximab; leflunomide; naproxen; valdecoxib; sulfasalazine; methylprednisolone; ibuprofen; meloxicam; methylprednisolone acetate; gold sodium thiomalate; aspirin; azathioprine; triamcinolone acetonide; propxyphene napsylate/apap; folate; nabumetone; diclofenac; piroxicam; etodolac; diclofenac sodium; oxaprozin; oxycodone hcl; hydrocodone bitartrate/apap; diclofenac sodium/misoprostol; fentanyl; anakinra, human recombinant; tramadol hcl; salsalate; sulindac; cyanocobalamin/fa/pyridoxine; acetaminophen; alendronate sodium; prednisolone; morphine sulfate; lidocaine hydrochloride; indomethacin; glucosamine sulfate/chondroitin; cyclosporine; amitriptyline hcl; sulfadiazine; oxycodone hcl/acetaminophen; olopatadine hcl; misoprostol; naproxen sodium; omeprazole; mycophenolate mofetil; cyclophosphamide; rituximab; IL-1 TRAP; MRA; CTLA4-IG; IL-18 BP; ABT-874; ABT-325 (anti-IL 18); anti-IL 15; BIRB-796; SCIO-469; VX-702; AMG-548; VX-740; Roflumilast; IC-485; CDC-801; and mesopram. In another embodiment, the TNFα antibody of the invention is administered for the treatment of a coronary disorder in combination with one of the above mentioned agents for the treatment of rheumatoid arthritis.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of a coronary disorder in

which TNFα activity is detrimental: anti-IL12 antibody (ABT 874); anti-IL18 antibody (ABT 325); small molecule inhibitor of LCK; small molecule inhibitor of COT; anti-IL1 antibody; small molecule inhibitor of MK2; anti-CD19 antibody; small molecule inhibitor of CCR5; and small molecule inhibitor of CXCR3.

In yet another embodiment, the TNFα antibody of the invention is administered in combination with an antibiotic or antiinfective agent. Antiinfective agents include those agents known in the art to treat viral, fungal, parasitic or bacterial infections. The term, "antibiotic," as used herein, refers to a chemical substance that inhibits the growth of, or kills, microorganisms. Encompassed by this term are antibiotic produced by a microorganism, as well as synthetic antibiotics (e.g., analogs) known in the art. Antibiotics include, but are not limited to, clarithromycin (Biaxin®), ciprofloxacin (Cipro®), and metronidazole (Flagyl®).

In one embodiment the TNFα inhibitor is administered following an initial procedure for treating coronary heart disease. Examples of such procedures include, but are not limited to coronary artery bypass grafting (CABG) and Percutaneous transluminal coronary balloon angioplasty (PTCA) or angioplasty. In one embodiment, the TNFα inhibitor is administered in order to prevent stenosis from re-occurring. In another embodiment of the invention, the TNFα inhibitor is administered in order to prevent or treat restenosis. The invention also provides a method of treatment, wherein the TNFα inhibitor is administered prior to, in conjunction with, or following the insertion of a stent in the artery of a subject receiving a procedure for treating coronary heart disease. In one embodiment the stent is administered following CABG or PTCA. A wide variety of stent grafts may be utilized within the context of the present invention, depending on the site and nature of treatment desired. Stent grafts may be, for example, bifurcated or tube grafts, cylindrical or tapered, self-expandable or balloon-expandable, unibody, or, modular. Moreover, the stent graft may be adapted to release the drug at only the distal ends, or along the entire body of the stent graft. The TNFa inhibitor of the invention can also be administered on a stent. In one embodiment, the TNFα antibody of the invention, including, for example, D2E7/HUMIRA® is administered by a drug-eluting stent.

The TNFα antibody of the invention can be administered in combination with an additional therapeutic agent to treat restenosis. Examples of agents which can be used to treat or prevent restenosis include sirolimus, paclitaxel, everolimus, tacrolimus, ABT-578, and acetaminophen.

The TNFα antibody of the invention can be administered in combination with an additional therapeutic agent to treat myocardial infarction. Examples of agents which can be used to treat or prevent myocardial infarction include aspirin, nitroglycerin, metoprolol tartrate, enoxaparin sodium, heparin sodium, clopidogrel bisulfate, carvedilol, atenolol, morphine sulfate, metoprolol succinate, warfarin sodium, lisinopril, isosorbide mononitrate, digoxin, furosemide, simvastatin, ramipril, tenecteplase, enalapril maleate, torsemide, retavase, losartan potassium, quinapril hcl/mag carb, bumetanide, alteplase, enalaprilat, amiodarone hydrochloride, tirofiban hcl m-hydrate, diltiazem hydrochloride, captopril, irbesartan, valsartan, propranolol hydrochloride, fosinopril sodium, lidocaine hydrochloride, eptifibatide, cefazolin sodium, atropine sulfate, aminocaproic acid, spironolactone, interferon, sotalol hydrochloride, potassium chloride, docusate sodium, dobutamine hcl, alprazolam, pravastatin sodium, atorvastatin calcium, midazolam hydrochloride, meperidine hydrochloride, isosorbide dinitrate, epinephrine, dopamine hydrochloride, bivalirudin, rosuvastatin, ezetimibe/simvastatin, avasimibe, abciximab, and cariporide.

The TNFα antibody of the invention can be administered in combination with an additional therapeutic agent to treat angina. Examples of agents which can be used to treat or prevent angina include: aspirin; nitroglycerin; isosorbide mononitrate; metoprolol succinate; atenolol; metoprolol tartrate; amlodipine besylate, dilitiazem hydropchloride, isosorbide dinitrate; clopidogrel bisulfate; nifedipine; atorvastatin calcium; potassium chloride; furosemide; simvastatin; verapamil hcl; digoxin; propranolol hcl; carvedilo; lisinopril; sprionolactone; hydrochlorothiazide; enalapril maleate; madolol; ramipril; enoxaparin sodium; heparin sodium; valsartan; sotalol hydrochloride; fenofibrate; ezetimibe; bumetanide; losartan potassium; lisinopril/hydrochlorothiazide; felodipine; captopril; and bisoprolol fumarate.

Any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from a coronary disorder in which TNFα is detrimental in combination with the TNFα antibody of the invention. In one

embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to a TNFα antibody to treat a coronary disease, including a restenosis. In another embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered in combination with the TNFα antibody of the invention, to a subject suffering from an coronary disorder, such as restenosis.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference

EXAMPLES

Example 1: TNFα Inhibitor In Reducing Inflammation and Restenosis

Study of restenosis using mouse carotid artery model

The following study of restenosis is performed using the mouse carotid artery model (Kumar and Lindner (1997) *Arterioscler. Thromb. Vasc. Biol.* 17:2238; de Waard *et al.* (2002) *Arterioscler. Thromb. Vasc. Biol.* 22:1978). Mice, ranging in age from two to four months, are anesthetized by intraperitoneal (i.p.) injection of a solution of xylazine. The left common carotid artery is dissected and ligated near the carotid bifurcation. Mice are then allowed to recover.

A monoclonal anti-TNFα antibody which is known to bind and neutralize mouse TNFα, *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et* al. (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) is administered to the experimental group. Mice receive daily subcutaneous injections per week of either the anti-TNF antibody or a placebo. At either 2.5 or 4 weeks after the ligation of the carotid artery, mice are sacrificed and subsequently fixed by perfusion with 4% paraformaldehyde in PBS. The carotid arteries are excised, immersed in 70% (v/v) ethanol, and embedded in paraffin. The nonligated right carotid artery serves as an

internal control for both the D2E7 injected and placebo injected mice. Serial sections are cut for morphometric analysis, as described in de Waard *et al., supra.*

Morphometric analysis provides a measurement of the total vessel area for the treated and untreated ligated carotids at certain set distances from a common physical reference point. It has previously been shown that the ligation results in the narrowing of the arteries (constructive remodeling) (Kumar and Lindner, *supra*; Kumar *et al.* (1997) *Circulation* 96:4333). Cross sections of the carotids are mounted on microscopic slides and stained with hematoxylin and eosin. Images of the carotid arteries are obtained using microscopic digital photography and the cross sectional areas of the intimal and the media are measured for a decrease in arterial narrowing (i.e, larger vessel diameter) as compared to placebo injected mice.

Example 2 : TNFα Inhibitor in Monkey Model of Atherosclerosis

Effect of D2E7 in monkey model of atherosclerosis.

The following study is performed using a diet-induced monkey model of atherosclerosis (Lentz SR *et al.* (2002) *Circulation* 106(7):842-6; Sundell CL *et al.* (2003)305(3):1116-23).

Adult cynomolgus monkeys (*Macaca fascicularis)* are fed an atherogenic diet that contains 0.7% cholesterol and 43% total calories as fat. After 44± 1 months on the atherogenic diet, animals are sedated with ketamine hydrochloride (20mg/kb IM) and anesthetized with sodium pentobarbital (20mg/kg IV). A nonobstructive catheter is inserted into an axillary artery for blood sampling, and the axillary vein is cannulated for administration of either D2E7 or placebo and supplemental anesthesia (sodium pentobarbital 5mg/kg per hour). D2E7 has been shown to effectively inhibit TNFα activity in a variety of species, including cynomolgus monkeys (see U.S. Patent No. 6,258,562).

Prior to infusion of D2E7 or placebo, blood is collected from the axillary artery catheter directly into a 1/10 volume of 3.8% sodium citrate for hemostatic assaying. After collection, blood samples are placed immediately on ice, and plasma is isolated by centrifugation at 2500g for 30 minutes at 4°C. Additional blood samples are collected

into serum separator tubes for determination of cholesterol or into serum separator tubes prepared with 3.4 mmol/L EDTA for determination of total plasma homocysteine (tHCY).

D2E7 or placebo is infused in 10ml of saline over 10 minutes through the axillary vein catheter. After infusion, blood samples are collected regularly.

The degree to which the animals are suffering atherosclerosis after treatment is assessed in various ways. Serum samples are regularly taken from the monkeys and assayed for total cholesterol, HDL cholesterol, LDL cholesterol, tHCY and triglycerides. Treated monkeys are examined to determine if total cholesterol, and LDL cholesterol, and tHCY levels are lower as compared to placebo treated monkeys, and whether HDL levels are higher.

Example 3: TNFα Inhibitor on Treating Restenosis in Patients

Study of D2E7 in human subjects with restenosis

Patients who have undergone balloon angioplasty are chosen for the study, as they have an increased chance of restenosis occurring within the first six months following angioplasty.

Prior to treatment, estimates of vessel and lesion parameters are made with reference to the guiding catheter. Estimates include reference vessel diameter (RVD), pretreatment minimal luminal diameter (MLD, which is determined by (RVD X [1 – preprocedural percent diameter stenosis]), postprocedural MLD (which is determined by (RVD X [1 – postprocedural percent diameter stenosis]), acute gain (postprocedural MLD – preprocedural MLD), number of diseased vessels and number of traded vessels.

Experimental group of patients are administered either D2E7 in biweekly and weekly doses of 40 mg or a placebo. Dosages may be adjusted by an ordinarily skilled artisan knowledgeable in restenosis. Patients are following and assessed at six months post-angioplasty to determine whether restenosis has occurred. Patients are also assessed at 9 months and long-term to determine the effect of delayed restenosis in those groups where restenosis was prevented or reduced due to treatment. Estimates of vessel and lesion parameters are recorded following D2E7 treatment. Statistical analysis is

performed to compare the extent of restenosis in the patients. (Jackson *et al.* (2003) *Am Heart J* 145:875).

<u>Example 4: TNFα Inhibitor on Treating Heart Failure</u>

Clinical study of D2E7 in human subjects with heart failure

Patients with stable New York Association (NYHA) class II or IV heart failure and left ventricular ejection fraction of less than 35% are chosen for the study. Under the NYHA standard, class III patients are defined as those with marked limitation of activity, *i.e.,* they are comfortable only at rest, and class IV patients are defined as those who should be at complete rest, *i.e.,* confined to bed or chair, or where any physical activity brings on discomfort and symptoms occur at rest. As described in Burns *et al.*, left ventricular ejection fraction is associated with six-month mortality (Burns *et al.* (2002) *J Am Coll Cardiol.* 39:30).

Patients receive biweekly doses of D2E7 at 40 mg, or a dosage adjusted by an ordinarily skilled artisan knowledgeable in heart failure. The control group is given a placebo. Patients undergo examinations at 1, 2, 6, 10, 14, 20, and 18 weeks. At each visit, each patient is examined and given an assessment of their overall heart failure status, relative to their status at the onset of the study, *i.e.*, their NYHA class is assessed. At the end of the heart failure study, the patient's final NYHA class is compared to the initial NHYA class.

<u>Example 5: Crystallization of D2E7 F(ab)'$_2$ fragment</u>

Generation and purification of the D2E7 F(ab)'$_2$ Fragment

A D2E7 F(ab)'$_2$ fragment was generated and purified according to the following procedure. Two ml of D2E7 IgG (approximately 63 mg/ml) was dialyzed against 1 liter of Buffer A (20 mM NaOAc, pH 4) overnight. After dialysis, the protein was diluted to a concentration of 20 mg/ml. Immobilized pepsin (Pierce; 6.7 ml of slurry) was mixed with 27 ml of Buffer A, mixed, and centrifuged (Beckman floor centrifuge, 5000 rpm, 10 min). The supernatant was removed, and this washing procedure was repeated twice

more. The washed immobilized pepsin was re-suspended in 13.3 ml of Buffer A. D2E7 (7.275 ml, 20 mg/ml, 145.5 mg) was mixed with 7.725 ml of Buffer A Bnd 7.5 ml of the washed immobilized pepsin slurry. The D2E7/pepsin mixture was incubated at 37 °C for 4.5 hr with shaking (300 rpm). The immobilized pepsin was then separated by centrifugation. Analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was essentially complete (~115 kDa band unreduced, ~30 and ~32 kDa bands reduced).

The D2E7 F(ab)'$_2$ fragment was separated from intact D2E7 and Fc fragments using Protein A chromatography. One-half of the above reaction supernatant (10 ml) was diluted with 10 ml of Buffer B (20 mM Na phosphate, pH 7), filtered through a 0.45 μm Acrodisk filter, and loaded onto a 5 ml Protein A Sepharose column (Pharmacia Hi-Trap; previously washed with 50 ml of Buffer B). Fractions were collected. After the protein mixture was loaded, the column was washed with Buffer B until the absorbance at 280 nm re-established a baseline. Bound proteins were eluted with 5 ml of Buffer C (100 mM citric acid, pH 3); these fractions were neutralized by adding 0.2 ml of 2 M Tris•HCl, pH 8.9. Fractions were analyzed by SDS-PAGE; those that contained the D2E7 F(ab)'$_2$ fragment were pooled (~42 ml). Protein concentrations were determined by absorbance at 280 nm in 6 M guanidine•HCl, pH 7 (calculated extinction coefficients: D2E7, 1.39 (AU-ml)/mg; F(ab)'$_2$, 1.36 (AU-ml)/mg). The flow-though pool contained ~38.2 mg protein (concentration, 0.91 mg/ml), which represents a 79% yield of F(ab)'$_2$ (theoretical yield is ~2/3 of starting material, divided by two [only half purified], i.e. ~48.5 mg).

The D2E7 F(ab)'$_2$ fragment was further purified by size-exclusion chromatography. The pooled Protein A flow-through was concentrated from ~42 to ~20 ml, and a portion (5 ml, ~7.5 mg) was then chromatographed on a Superdex 200 column (26/60, Pharmacia) previously equilibrated (and eluted) with Buffer D (20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA). Two peaks were noted by absorbance at 280 nm: Peak 1, eluting at 172–200 ml, consisted of F(ab)'$_2$ (analysis by SDS-PAGE; ~115 kDa band unreduced, ~30 and ~32 kDa bands reduced); Peak 2, eluting at 236–248 ml, consisted of low molecular weight fragment(s) (~15 kDa, reduced or unreduced). Peak 1 was concentrated to 5.3 mg/ml for crystallization trials.

Crystallization of the D2E7 F(ab)'$_2$ Fragment

The D2E7 F(ab)'$_2$ fragment (5.3 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method by mixing equal volumes of F(ab)'$_2$ and crystallization buffer (approx. 1 μl of each) and allowing the mixture to equilibrate against the crystallization Buffer Bt 4 or 18 °C. The crystallization buffers used consisted of the Hampton Research Crystal Screens I (solutions 1–48) and II (solutions 1–48), Emerald Biostructures Wizard Screens I and II (each solutions 1–48), and the Jena Biosciences screens 1–10 (each solutions 1–24). Crystals were obtained under many different conditions, as summarized in Table 1.

Table 1. Summary of crystallization conditions for the D2E7 F(ab)'$_2$ fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	32	4	2.0 M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 1	46	4	0.2 M Ca(Oac)$_2$, 0.1 M Na cacodylate pH 6.5, 18% PEG 8K	medium sized needle clusters
Hampton 1	48	4	0.1 M Tris HCl pH 8.5, 2.0 M $NH_4H_2PO_4$	micro needle clusters
Hampton 2	2	4	0.01 M hexadecyltrimethylammonium bromide, 0.5 M NaCl, 0.01 M $MgCl_2$	small shard crystals
Hampton 2	13	4	0.2 M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.6, 30% PEG MME 2000	small needle clusters
Hampton 2	15	4	0.5 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	large needle clusters
Hampton 2	16	4	0.5M NaCl, 0.1M NaOAc pH 5.6, 4% Ethylene Imine polymer	large irregular crystal
Hampton 1	34	18	0.1 NaOAc pH 4.6, 2.0 M Na Formate	needle clusters
Hampton 1	35	18	0.1M Hepes pH 7.5, 0.8M mono-sodium dihydrogen phosphate, 0.8M mono-potasium dihydrogen phosphate	needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2.0M NaCl	dense needle clusters
Hampton 2	12	18	0.1M $CdCl_2$, 0.1M NaOAc pH 4.6, 30% PEG 400	needles & amorphous crystals
Hampton 2	15	18	0.5M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	tiny needle clusters
Wizard I	27	4	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Medium large needle clusters
Wizard I	30	4	1.26M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.5, 0.2M NaCl	small needle clusters
Wizard II	8	4	10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl	Large plate crystals grown in clusters
Wizard II	43	4	10% PEK 8K, 0.1M Tris pH 7.0, 0.2 M MgCl2	micro needle clusters
Wizard I	4	18	35% MPD, 0.1M Imidazole pH 8.0, 0.2M MgCl2	rod shaped crystal
Wizard I	27	18	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Needle clusters
Wizard II	7	18	30% PEG 3K, 0.1M Tris pH 8.5, 0.2M NaCl	tiny needle clusters
Wizard II	11	18	10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)2	tiny hexagonal or rhombohedral crystals

Screen	Solution	Temp °C	Condition	Result
Wizard II	46	18	1.0M AP, 0.1M Imidazole pH 8.0, 0.2M NaCl	1 irregular crystal
JB 1	D6	4	30% PEG 3K, 0.1M Tris HCl pH 8.5, 0.2M Li_2SO_4	tiny needles in precipitate
JB 2	B6	4	20% PEG 4K, 0.1M Tris HCl pH 8.5, 0.2M Na Cacodylate	tiny needle cluster balls
JB 3	A1	4	8% PEG 4K, 0.8M LiCl, 0.1M Tris HCl pH 8.5	Large frost-like crystals
JB 3	B1	4	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	tiny needle clusters
JB 3	D5	4	30% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	tiny needles in precipitate.
JB 4	B1	4	15% PEG 6K, 0.05M KCl, 0.01M $MgCl_2$	needle cluster balls
JB 3	A6	18	12% PEG 4K, 0.1M NaOAc pH 4.6, 0.2M NH_4OAc	needle clusters
JB 3	B1	18	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	needle clusters in precipitate
JB 3	C6	18	25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	long, thin needles
JB 4	C5	18	8% PEG 8K, 0.2 M LiCl, 0.05M $MgSO_4$	frost-like crystals
JB 5	A3	4	15% PEG 8K, 0.2M $(NH_4)_2SO_4$	long single needles in phase separation
JB 5	A4	4	15% PEG 8K, 0.5M Li_2SO_4	tiny needle clusters
JB 5	A5	4	15% PEG 8K, 0.1M Na MES pH 6.5, 0.2M $Ca(OAc)_2$	needle cluster balls
JB 6	B2	4	1.6M $(NH_4)_2SO_4$, 0.5 LiCl	tiny needle cluster balls
JB 6	C2	4	2.0 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6	micro needle clusters
JB 10	D3	18	2.0M Na Formate, 0.1M NaOAc pH 4.6	needle clusters

The following conditions (as described in Table 1) produced crystals which can be used for diffraction quality crystals: Wizard II, 11, 18, 10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)2, tiny hexagonal or rhom. Xtals; Wizard II, 10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl, large plate xtals grown in clusters; JB 3, C6, 18, 25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M Ammonium Acetate, long, thin needles; Hampton 2, 15, 18, 0.5M AS, 0.1M Na Acetate trihydrate pH 5.6, 1.0M Li Sulfate monohydrate, tiny needle clusters.

Example 6: Crystallization of D2E7 Fab fragment

Generation and purification of the D2E7 Fab Fragment

A D2E7 Fab fragment was generated and purified according to the following procedure. Four ml of D2E7 IgG (diluted to about 20 mg/ml) was diluted with 4 ml of Buffer E (20 mM Na phosphate, 5 mM cysteine•HCl, 10 mM EDTA, pH7) and mixed with 6.5 ml of a slurry of immobilized papain (Pierce, 1%; previously washed twice with

26 ml of Buffer E). The D2E7/papain mixture was incubated at 37 °C overnight with shaking (300 rpm). The immobilized papain and precipitated protein were separated by centrifugation; analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was partially complete (~55, 50, 34, and 30 kDa bands unreduced, with some intact and partially digested D2E7 at ~115 and ~150 kDa; ~30 and ~32 kDa bands reduced, as well as a ~50 kDa band). Nonetheless, the digestion was halted and subjected to purification.

The D2E7 Fab fragment was purified by Protein A chromatography and Superdex 200 size-exclusion chromatography essentially as described above for the F(ab)'$_2$ fragment. The Protein A column flow-through pool (21 ml) contained ~9.2 mg (0.44 mg/ml), whereas the Protein A eluate (4 ml) contained ~19.5 mg (4.9 mg/ml). Analysis by SDS-PAGE indicated that the flow-through was essentially pure Fab fragment (~48 and ~30 kDa unreduced, broad band at ~30 kDa reduced), whereas the eluate was intact and partially-digested D2E7. The Fab fragment was further purified on a Superdex 200 column, eluting at 216–232 ml, i.e., as expected, after the F(ab)'$_2$ fragment but before the small Fc fragments. The D2E7 Fab fragment concentrated to 12.7 mg/ml for crystallization trials, as described below.

Crystallization of the D2E7 Fab Fragment

The D2E7 Fab fragment (12.7 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method essentially as described above for the F(ab)'$_2$ fragment. Crystals were obtained under many different conditions, as summarized in **Table 2**.

Table 2. Summary of crystallization conditions for the D2E7 Fab fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	4	4	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	wispy needles
Hampton 1	10	4	0.2M NH_4OAc, 0.1M NaOAc pH 4.6, 30% PEG 4K	wispy needle clusters
Hampton 1	18	4	0.2M $Mg(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 20% PEG 8K	needle clusters
Hampton 1	20	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG 4K	tiny needle clusters
Hampton 1	32	4	2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 1	33	4	4M Na Formate	tiny needle clusters
Hampton 1	38	4	0.1M Hepes pH 7.5	tiny needle clusters
Hampton 1	43	4	30% PEG 1500	tiny needle clusters

Screen	Solution	Temp °C	Condition	Result
Hampton 1	46	4	0.2M $Ca(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 18% PEG 8K	large plate clusters
Hampton 1	47	4	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 2	1	4	2M NaCl, 10% PEG 6K	small plate clusters
Hampton 2	2	4	0.01M Hexadecyltrimethylammonium bromide, 0.5M NaCl, 0.01 $MgCl_2$	round & irregular plates
Hampton 2	5	4	2M $(NH_4)_2SO_4$, 5% isopropanol	long fiber ropes
Hampton 2	13	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny, wispy needle clusters
Hampton 2	14	4	0.2M K/Na Tatrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	27	4	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Hampton 2	28	4	30% MPD	tiny needle clusters
Hampton 1	4	18	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	9	18	0.2M NH_4OAc, 0.1M Na Citrate pH 5.6, 30% PEG 4K	needle clusters
Hampton 1	17	18	0.2M Li_2SO_4, 0.1M Tris pH 8.5, 30% PEG 4K	long, wispy needles
Hampton 1	32	18	2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	33	18	4M Na Formate	tiny needle clusters
Hampton 1	38	18	0.1M Hepes pH 7.5	fiber bundles
Hampton 1	43	18	30% PEG 1500	tiny needle clusters
Hampton 1	47	18	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	1	18	2M NaCl, 10% PEG 6K	long, wispy needle clusters
Hampton 2	5	18	2M $(NH_4)_2SO_4$, 5% 2-propanol	tiny needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2M NaCl	long, wispy needles
Hampton 2	13	18	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny needle clusters
Hampton 2	14	18	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	long wispy needles
Hampton 2	27	18	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Wizard I	20	4	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	28	4	20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl	large orthorhombic plate clusters
Wizard I	31	4	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	wispy needle clusters
Wizard I	39	4	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	3	4	20% PEG 8K, 0.1M Tris pH 8.5, 0.2M $MgCl_2$	large hexagonal or orthorhombic plate cluster in phase sep
Wizard II	4	4	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	tiny needle clusters
Wizard II	9	4	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	tiny, wispy needle clusters
Wizard II	28	4	20% PEG 8K, 0.1M MES pH 6, 0.2M $Ca(OAc)_2$	tiny needle clusters; large wispy needle clusters
Wizard II	35	4	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny fiber bundles
Wizard II	38	4	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	long wispy needles
Wizard II	47	4	2.5M NaCl, 0.1M Imidazole pH 8, 0.2M $Zn(OAc)_2$	tiny needle clusters

Screen	Solution	Temp °C	Condition	Result
Wizard I	6	18	20% PEG 3K, 0.1M Citrate pH 5.5	needle clusters
Wizard I	20	18	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	27	18	1.2M NaH_2PO_4/0.8M K_2HPO_4, 0.1M CAPS pH 10, 0.2M Li_2SO_4	wispy needle clusters
Wizard I	30	18	1.26M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.5, 0.2M NaCl	wispy needles
Wizard I	31	18	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	tiny needle clusters
Wizard I	33	18	2M $(NH_4)_2SO_4$, 0.1M CAPS pH 10.5, 0.2M Li_2SO_4	fiber bundles
Wizard I	39	18	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	4	18	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	needle clusters
Wizard II	9	18	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	wispy needles
Wizard II	35	18	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny needle clusters
Wizard II	38	18	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	tiny needle clusters

The following conditions (as described in Table 2) produced crystals which can be used for diffraction quality crystals: Hampton 2, 1, 4C, 2M NaCl, 10% PEG 6K, small plate clusters; Hampton 1 46, 4C, 0.2M Ca Acetate, 0.1M Na Cacodylate, pH 6.5, 18% PEG 8K, large plate clusters; Wizard I, 28, 4C, 20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl, large orthorhombic plate clusters; Wizard II 3, 4C, 20% PEG 8K, 0.1M Tris pH 8.5, 0.2M $MgCl_2$, lrg hex or orth plate cluster in phase sep.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.